Recent Progress on the Cytokine Regulation of Intestinal Immune Responses to *Eimeria*[1]

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ABSTRACT A variety of methods are available to combat avian diseases in the commercial setting, including improved farm management practices, use of antibiotic drugs, selection of disease resistant chicken strains, and manipulation of the chicken immune system. In the latter category, development of vaccines against the major avian diseases has become a priority for the poultry industry. With increasing demands for developing alternative control programs for many poultry diseases, it is important to understand the basic immunobiology of host-pathogen interactions in order to develop novel vaccination strategies. From studies carried out in many mammalian species, it is evident that host immune responses to intracellular pathogens are complex and involve many components of the host immune system. For enteric pathogens such as *Eimeria* and *Salmonella*, understanding cell-mediated immunity is most important because antibodies, although abundantly produced locally, can not access and act on these intracellular pathogens. In poultry, slow but increasing understanding of various components of host immune system mediating cellular immunity is opening new opportunities for thorough investigation of the role of thymus-derived lymphocyte subpopulations and cytokines in normal and disease states. This paper will review recent progress with chicken cytokines that have been characterized, and discuss various experimental strategies to enhance host immunity to pathogens using chicken cytokines.

(*Key words*: chicken, cytokine, *Eimeria*, immunity, vaccine)

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INTRODUCTION

The major parasitic disease of poultry, coccidiosis, causes more than $800 million in losses in prophylactic medication (Williams, 1998), and this number is expected to rise due to the increasing regulatory restrictions on the use of traditionally used drugs for coccidiosis control. Economic losses due to coccidiosis include mortality, malabsorption, inefficient feed utilization, and impaired growth rate in broilers and a temporary reduction of egg production in layers. Coccidia parasites are distributed worldwide. Because the life cycle of *Eimeria* comprises intracellular, extracellular, asexual, and sexual stages, immune responses to *Eimeria* are complex and involve many facets of nonspecific and specific immunity, the latter encompassing both cellular and humoral immune mechanisms (Lillehoj and Lillehoj, 2000; Lillehoj and Okamura, 2003). Chickens, when they survive the initial infection, become resistant to reinfection due to the development of a protective immunity that normally lasts for at least several months (Rose, 1976). In the natural host, the immunity is species specific, such that chickens immune to one species of *Eimeria* are susceptible to others. Additionally, the *Eimeria* spp. demonstrate different tissue and organ specificities in the infected host. Understanding the interplay between the host and the parasites in the intestine is thus crucial for designing new control approaches against coccidiosis. Development of novel immunological, genetic, and molecular strategies is needed not only for identification of the *Eimeria* immunogens but also for the general understanding of the basic immunobiology of host protective immunity to intracellular pathogens in the intestine.

AVIAN GUT IMMUNE SYSTEM

The gut-associated lymphoid tissues (GALT) represent a component of the mucosa-associated lymphoid tissues,

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Abbreviation Key: ConA = concanavalin A; GALT = gut-associated lymphoid tissues; IEL = intraepithelial lymphocytes; mAb = monoclonal antibody; MIP-1β = mammalian macrophage inflammatory protein 1β; NK = natural killer; NO = nitric oxide; ORF = open reading frame; PBL = peripheral blood lymphocytes; RT = reverse transcription; SCID = severe combined immune-deficient; TCR = T-cell receptor.

which, in mammals, also include the bronchial organs, salivary glands, nasopharyngeal tracts, genitourinary tracts, and mammary glands. The chicken immune system shares similar anatomical and functional organizations with its mammalian counterpart and includes cellular and humoral immune components. Shared features are the analogous localization of thymus, spleen, lymph nodes, Peyer's patches, intestinal epithelial lymphocytes, and Harderian gland. Differences, however, do exist between birds and higher vertebrates. One of the most anatomically prominent features in the chicken immune system is the presence of the bursa of Fabricius at the dorsal side of the proctodaeal region of the cloaca. This is a central organ for the development of B cells and provides an essential microenvironment for the expansion of the B-cell pool and the generation of a diversified B-cell repertoire. Other unique features include Meckel's diverticulum, a remnant of the yolk sac and stalk located in the middle of the small intestine, the cecal tonsils as lymphoid tissues in the proximal region of the ceca, and the pineal gland as a lymphomyeloid tissue (Cogburn and Glick, 1983). Because the invasive form of *Eimeria* accesses the intestinal mucosae as its portal of entry into the host, the chicken GALT play a decisive role as the third line of defense as well as in the subsequent host immune protection. Shortly after infection with *Eimeria* spp., the intestinal B cells begin producing parasite-specific antibodies, although their role in host defense against *Eimeria* is debatable. However, extensive experimental evidence supports the notion that cell-mediated immunity, predominantly mediated by the intestinal intraepithelial and lamina propria lymphocytes, represents the main component of protective immunity to avian coccidiosis.

The GALT of chickens consist of the bursa of Fabricius, cecal tonsils, lymphocyte aggregates in the epithelium and the lamina propria of the intestinal wall, and Peyer's patches (Befus et al., 1980). The GALT contain B and T lymphocytes responsible for acquired immunity to enteric pathogens (Lillehoj and Lillehoj, 2000), macrophages, and natural killer (NK) cells, important mediators of host innate immunity (Lillehoj and Chai, 1988). The gastrointestinal epithelium is covered by a protective mucus gel composed predominantly of mucin glycoproteins that are synthesized and secreted by goblet cells. The mucous layer functions as a physical barrier against intruding pathogens. Many other factors present in the intestinal tract can function as nonspecific defense barriers, and these include gastric secretion, lysozymes, bile salts, microbial flora, and endogenous cationic peptides such as defensins. Gallinacin-3 is a recently described chicken defensin present predominantly in the tongue, bursa of Fabricius, and trachea (Zhao et al., 2001); it functions in killing microorganisms. Four avian β-defensin gallinacins, Gal 1 (CHP 1, chicken heterophil peptide 1), Gal 2, and turkey heterophil peptides, THP 1 and THP 2, have been characterized (Evans et al., 1994, 1995; Brockus et al., 1998). Although these antimicrobial peptides and proteins are an integral part of the epithelial defense barrier that provides immediate protection against bacterial invasion, the precise role of defensins in the local defense against coccidia has not been studied.

HUMORAL IMMUNE RESPONSES TO *EIMERIA*

As in mammals, 3 principal classes of antibodies are known in birds, IgM, IgA, and IgY. Each antibody class has at least one monomer of heavy chains and light chains. Both heavy and light chains have a variable domain at the N-terminal and 3 or 4 constant domains at the C-terminal. Depending on the types of constant regions of the heavy (H) chains, the antibodies are classified into IgM (μH chain), IgA (αH chain), and IgY (γH chain). Although IgY is considered the orthologue of the mammalian IgG (Leslie and Clem, 1969), the cDNA encoding the IgY heavy chain has similarity to mammalian IgE (Parvari et al., 1988). The presence of other antibody classes such as IgD or IgE in birds has not been documented.

Chickens infected with *Eimeria* produce parasite-specific antibodies in both the systemic circulation and the mucosal secretions (Lillehoj and Trout, 1996). Upon exposure to *Eimeria* spp., chickens produce all 3 classes of antibodies. IgY, equivalent to the mammalian IgG, is produced by lower vertebrates such as reptiles, amphibians, and birds. This class of antibody is concentrated in the egg yolk in laying hens and is thus considered to be of some relevance in maternal immunity (Rose and Long, 1971; Rose, 1972; Smith et al., 1994a,b; Wallach, 1997). Wallach et al. (1992) used gametocyte surface antigens of *E. maxima* to immunize laying hens for the passive transfer of secretory antibodies. Upon challenge with sporulated *E. maxima* oocysts, the chicks from the hens immunized with the affinity-purified gametocyte antigens showed a greatly reduced oocyst production as compared with the chicks from the sham-immunized hens. This transmission-blocking immunity against the gametocyte stage of the parasite, mediated by the maternally transferred antibodies, was effective in reducing the sexual reproduction of oocysts but did not prevent the pathogenic effects of sporozoites or merozoites.

CELL-MEDIATED IMMUNE RESPONSES

The evidence that the removal of the bursa by chemical or hormonal means (Rose and Long, 1970; Giambrone et al., 1981; Lillehoj, 1987) does not interfere with the development of protective immunity against *Eimeria* indicates the importance of cell-mediated immunity in coccidiosis. The role of T cells in the protection against coccidiosis has also been studied in immunosuppressed chickens by using T-cell-specific drugs that selectively abrogate or severely impair the T-cell function. These treatments included thymectomy (Rose and Long, 1970), cyclosporin A (Lillehoj, 1987), betamethasone, dexamethasone (Isobe and Lillehoj, 1993), and monoclonal antibodies (mAb) against CD8+ or $\alpha\beta$ T-cell receptor (TCR)-

expressing cells (Trout and Lillehoj, 1996). In all of these studies, the abrogation of T-cell function impaired the host-protective immunity against coccidiosis. Additional evidence for the protective role of T cells came from the adoptive transfer studies where peripheral blood lymphocytes (PBL) and spleen cells from *E. maxima*-immune chickens protected syngeneic recipients against a live parasite challenge infection (Rose and Hesketh, 1982). Miller et al. (1994), using an in vitro culture system, showed that splenocytes from *Eimeria tenella*-immune chickens inhibited the intracellular development of *E. tenella* in chicken kidney cells. The nature of these cells was not determined, but these cells may represent NK cells because they did not show any MHC restriction in their action. Direct evidence for the presence of *Eimeria*-specific T cells was demonstrated by an in vitro antigen-driven lymphoproliferation assay (Rose and Hesketh, 1984; Lillehoj, 1986; Vervelde et al., 1996). T lymphocytes specifically reactive to *E. tenella* antigens were cloned by continuous in vitro culture in the presence of crude culture supernatant containing a T-cell growth factor (Bhogal et al., 1986).

T-Cell Subset Responses

The intraepithelial lymphocytes (IEL) represent an important component of the GALT (Guy-Grand et al., 1974). A unique feature of IEL is that $\gamma\delta$ T cells are predominant whereas the vast majority of mature T lymphocytes in the peripheral blood and lymphoid organs use the CD3-associated $\alpha\beta$ TCR heterodimer for antigen recognition (Bonneville et al., 1988; Goodman and Lefrancois, 1988). After primary and secondary infections with *Eimeria acervulina*, an increased percentage of intraepithelial $\gamma\delta$ T cells was observed in the duodenum (Choi and Lillehoj, 2000). The percentage of $\gamma\delta$ T cells was significantly elevated by d 8 following primary infection with *E. acervulina* in SC chickens, an inbred B^2B^2 line, whereas a significant increase was observed as early as 4 d in TK chickens, an inbred $B^{15}B^{21}$ line (Choi et al., 1999). Concurrent with the increase of $\gamma\delta$ T cells, a significant enhancement of interleukin (IL)-2 mRNA transcripts was found (Choi and Lillehoj, 2000). The percentage of $\alpha\beta$ T cells was elevated in IEL by d 4 after primary infection with *E. acervulina* in SC chickens, whereas a significant increase of $\alpha\beta$ T cells was not observed until 6 d postsecondary infection in SC chickens (Choi et al., 1999).

The importance of CD8+ T cells has been shown in many intracellular parasitic infections including toxoplasmosis (Hakim et al., 1991) and malaria (Weiss et al., 1990). In avian coccidiosis, the selective elimination of CD8+ cells by anti-CD8+ mAb resulted in exacerbation of disease as evidenced by increased oocyst shedding after infection with *E. tenella* or *E. acervulina* (Trout and Lillehoj, 1996). Significant increase of T cells expressing CD8+ molecules was noted in the intestinal IEL population following the challenge infections of chicken with *E. acervulina* (Lillehoj and Bacon, 1991). When 2 MHC-congenic chickens with a different disease susceptibility to coccidiosis were compared, the higher increase of $\alpha\beta$ TCR+CD8+ and $\gamma\delta$ TCR+CD8+ cells was associated with B^2B^2 chickens, which are less susceptible. Similarly, Bessay et al. (1996) observed a significant increase in the proportion of CD4+, CD8+, and TCR $\gamma\delta$ in the duodenal IEL from d 4 to 8 postinfection with *E. acervulina*. In contrast, the proportion of CD8+ cells decreased significantly in the blood and spleen on d 4 and 6 postinfection. After *E. tenella* infection, the proportion of CD4+ cells in the cecal IEL increased on d 8 postinfection, and CD8+ cells increased on d 6 and 8 postinfection. At the same time, the proportion of CD4+ cells decreased in the spleen on d 8 postinfection, and CD8+ cells decreased in the blood on d 6.

In the peripheral blood, a transient but sharp increase in the proportion of CD8-expressing T cells was found in White Leghorn chickens at 8 d after a primary infection with *E. tenella* (Breed et al., 1996, 1997a,b). This increase was found to be concurrent with a marked increase in interferon (IFN)-γ and in nitric oxide (NO) production upon in vitro stimulation of PBL by T-cell mitogens and *E. tenella* sporozoite antigen (Breed et al., 1997a). In *E. maxima* infection, both CD4+ and CD8+ cells were observed in the small intestine of Light Sussex chickens, but the proportion of CD8+ cells was higher (Rothwell et al., 1995). After a secondary infection with *E. acervulina*, a significant increase of CD8+ T cells in duodenum IEL occurred in SC chickens (Lillehoj and Bacon, 1991; Lillehoj, 1994). Two-color immunofluorescence revealed that the majority of CD8+ cells in the duodenum intraepithelium of immune chickens coexpressed $\alpha\beta$ TCR. In SC and TK chickens, the ratio of CD8+ to CD4+ T lymphocytes in IEL was elevated by d 4 following primary and secondary infections with *E. acervulina*. These cells continued to increase in SC chickens but showed a marked decrease in TK chickens following the secondary infection (Choi et al., 1999). In chickens infected with *Eimeria mivati*, the percentages of splenic lymphocytes bearing CD8+, $\gamma\delta$ TCR, class II MHC, or surface IgM antigens were decreased in the dexamethasone-treated chickens when compared with the normal chickens (Isobe and Lillehoj, 1993). Significantly higher numbers of total oocyst output in the dexamethasone-treated chickens following primary and secondary infections with *E. mivati* indicated the significance of CD8+ cells in primary as well as secondary immune responses.

CD4+ cells represent a minor population of the IEL. During *E. acervulina* infection, CD4+ cells increased 7 d after primary and 14 d after secondary infection (Lillehoj, 1994). Bessay et al. (1996) examined the T lymphocyte subsets in the intestine following *E. tenella* and *E. acervulina* infections. Following *E. acervulina* infection, a significant increase in the proportion of CD4+ was observed in duodenal IEL from d 4 to 8, and in the blood and spleen on d 8 postinfection. In *E. tenella* infection, CD4+ cells increased on d 8 postinfection in the cecal IEL but the proportion of CD4+ cells dropped in the spleen on d 8 postinfection. In another study, different kinetics of CD4+ changes was observed (Breed et al., 1996). A sharp de-

crease in PBL CD4+ T cells occurred in outbred White Leghorn chicken after *E. tenella* infection at 9 to 10 d after primary infection, but no significant change was seen after secondary infection. In the ceca, the number of CD4+ cells increased significantly 2 d after infection with *E. tenella* and, in immune chickens, mainly CD4+ and CD8+ T cells infiltrated the lamina propria (Vervelde et al., 1996). A significantly higher number of sporozoites were found within or next to CD3+, CD8+, and $\alpha\beta$ TCR+ cells in immune chickens. Interestingly, a profound increase in the proportion of CD8+ cells preceded the sharp decrease in CD4+ cells (Breed et al., 1996). In a study aimed at elucidating the immunologic differences between resistant SC (B^2B^2) and susceptible TK ($B^{15}B^{21}$) chickens, the duodenal CD4+ T lymphocytes increased significantly and rapidly early (4 d) after primary and secondary infections with *E. acervulina* in SC as compared with TK chickens (Choi et al., 1999). The role of CD4+ T cells in coccidiosis may involve the production of soluble cytokines such as IFN-γ (Yun et al., 2000a,b,c). Using a quantitative reverse transcription (RT)-PCR, increased IFN-γ mRNA expression was observed in the cecal tonsil lymphocytes in *E. tenella*-infected SC chickens and the selective depletion of CD4+, but not CD8+, reduced IFN-γ production.

Non-T Cells

Role of NK cells in parasitic diseases has been well documented. NK-cell activity of peripheral blood from a group of Kenyan adults and children with acute *Plasmodium falciparum* malaria on erythrocytic schizonts of *P. falciparum* was examined in comparison with those of 3 d age- and sex-matched control cohorts: parasitaemic but asymptomatic children, aparasitaemic children and adults, and adult Caucasians with no previous history of malaria (Orago and Facer, 1991). Both CD3-CD16+ and CD3-CD56+ NK cells from all patients and donors lysed erythrocytic schizonts, and this cytotoxicity was enhanced by the addition of recombinant IFN-γ or IL-2, notably with the CD3-CD56+ subset. The asymptomatic donors had the highest levels of CD3-CD56+ NK cells, which were also responsive to cytokine stimulation. There was a statistically significant quantitative and qualitative depression of the CD3-CD56+ subset in patients with acute malaria (Orago and Facer, 1991). These results illustrate a role for NK cells in the protection against malaria through the lysis of infected erythrocytes. The chicken gut IEL also contain subpopulations of cells that can mediate NK cell activities as demonstrated by the 4-h ^{51}Cr release assay using different tumor cell targets of avian tumors (Chai and Lillehoj, 1988). The NK-cell activity was higher in the jejunum and ileum than in the duodenum and cecum. The role of NK-cell activity has been examined in avian coccidiosis (Lillehoj, 1989). Following the infection with *Eimeria* parasites, the NK-cell activities of both splenic and intestinal IEL decreased to a subnormal level during the early phase of infection. NK-cell activity returned to normal or slightly higher than normal levels at about 1 wk after the primary inoculation. Significant increases in the splenic and intestinal IEL NK-cell activities were observed during the early phase of secondary infection. This increase in the IEL NK-cell activity shortly after secondary infection was accompanied by a substantial increase in the number of IEL expressing the asialo-GM1 antigen, a NK marker (Lillehoj, 1989).

Chicken NK cells, defined phenotypically as CD8+ cells lacking T- or B-lineage specific markers, constitute approximately 30% of CD8+ intestinal IEL but <1% of splenocytes or PBL (Gobel et al., 2001). Using the mAb 28-4, specific for CD8+CD3-IEL and a mAb for CD3, IEL were separated into CD3+ IEL T cells and the 28-4+ cells, both co-expressing the CD8 antigen. The 28-4+ IEL were able to lyse the NK-sensitive targets. These results define the 2 major phenotypically and functionally distinct IEL subpopulations, and imply an important role of NK cells in the mucosal immune system (Gobel et al., 2001). Chicken NK cells were identified using the mAb K-14 and K-108, which stained 6 to 17% of splenic lymphocytes, 11 to 14% of PBL, and fewer than 5% of thymic and bursal lymphocytes (Chung and Lillehoj, 1991).

Chicken macrophages, identified using the mAb K1, expressed the MHC Class II antigen (Kaspers et al., 1993). In *E. tenella*-immune chickens, more leukocytes were present in the lamina propria, and leukocytes infiltrated the ceca more rapidly than in the naive chickens (Vervelde et al., 1996). By immunocytochemical staining, most infiltrated leukocytes were macrophages and T cells. To examine the macrophages' role in the control of *Eimeria* infection, various in vitro systems were carried out. Chicken macrophages pretreated with the culture supernatants of concanavalin A (ConA)-stimulated spleen cells or of a reticuloendotheliosis virus-transformed chicken T-cell line exerted cytostatic effects on the growth of *E. tenella* sporozoites (Dimier et al., 1998). These inhibitory effects were overcome when the culture was supplemented with oxygen scavengers such as superoxide dismutase, D-mannitol, DABCO, benzoic acid, and L-histidine hydrochloride (Dimier-Poisson et al., 1999). Pretreatment of macrophages with culture supernatants of ConA-stimulated spleen cells induced NO synthesis, and the addition of NG monomethyl-L-arginine, a NO synthase inhibitor, also overcame the inhibition of *E. tenella* replication in macrophage cultures. Therefore, production of the inorganic NO or toxic oxygen intermediates may be involved in inhibiting *E. tenella* growth (Dimier-Poisson et al., 1999).

Heterophils are the predominant granulated leukocyte (comparable to mammalian neutrophils) seen during the acute inflammatory response in gallinaceous birds. Heterophils are highly phagocytic and are capable of a broad spectrum of antimicrobial activity. They accumulate in the inflamed tissue, causing tissue damage and forming heterophil granulomas that are morphologically similar to the inflammatory lesions in reptiles. The avian heterophils lack myeloperoxidase and depend primarily on the non-oxidative mechanisms for their antimicrobial activity. The β-defensins found in the heterophil granules can

TABLE 1. Cloned chicken genes of immunological importance

Cytokine/chemokine/other molecules	Gene	Accession no.	References
Interleukins			
Interleukin-1β	IL-1β	Y15006	Weining et al., 1998
Interleukin-2	IL-2	AF000631	Sundick and Gill-Dixon, 1997
Interleukin-6	IL-6	AJ309540	Schneider et al., 2001
Interleukin-8	IL-8	AJ009800	Kaiser et al., 1999
Interleukin 15	IL-15	AF139097	Lillehoj et al., 2002
Interleukin-16	IL-16	AJ508678	Min and Lillehoj, 2004
Interleukin-17	IL-17	AJ493595	Min and Lillehoj, 2002
Interleukin-18	IL-18	AJ277865	Schneider et al., 2000
Interferons			
Interferon-α	IFN-α	U07868	Sekellick et al., 1994
Interferon-β	IFN-β	X92479	Sick et al., 1996
Interferon-γ	IFN-γ	U27465	Digby and Lowenthal, 1995
Interferon type A1	IFNA1	X92476	Sick et al., 1996
Interferon type A2	IFNA2	X92477	Sick et al., 1996
Interferon type A3	IFNA3	X92478	Sick et al., 1996
Interferon type B	IFNB	X92479	Sick et al., 1996
Chemokines and other molecules			
Transforming growth factor-β2	TGF-β2	X59081	Burt and Paton, 1991
β-2 Microglobulin	β-2m	Z48922	Riegert et al., 1996
Caspase-1		AF031351	Johnson et al., 1998
Cysteine protease caspase-2	Ich-1	U64963	Johnson et al., 1997
Chemokine K203	K203	Y18692	Sick et al., 2000
CXC chemokine K60	K60	AF277660	Sick et al., 2000
Dorsalin-1		L12032	Basler et al., 1993
Macrophage inflammatory protein-1β	MIP-1β	L34553	Petrenko et al., 1995
Macrophage migration inhibitory factor	MIF	M95776	Wistow et al., 1993
Clone 391 cytokine		L34552	Petrenko et al., 1997
Glycoprotein 130	gp130	AJ011688	Geissen et al., 1998
Fibrogenic lymphokine fibrosin		AI981213	Tirunagaru et al., 2000
Natural resistance-associated macrophage protein 1	NRAMP1	S82465	Hu et al., 1996
α (1,3)-Fucosyltransferase	CFT1	U73678	Lee et al., 1996
β-Defensin	GAL2	AF033336	Brockus et al., 1998
Gal-1 α		AF181951	Zhao et al., 2001
Gallinacin 1	GAL1	AF033335	Brockus et al., 1998

kill a wide variety of bacterial pathogens and represent a major component of the heterophil antimicrobial arsenal (Harmon, 1998). Heterophils form the first line of cellular defense against the invading microbial pathogens. A few studies examined the response of heterophils during *Eimeria* infection in chickens. The ratios of heterophils to lymphocytes were increased in female Hubbard × Hubbard chicks during *E. acervulina* infection (McFarlane and Curtis, 1989). In contrast, female Hubbard × Hubbard broiler chicks showed lower ratios after infection with *E. tenella* (McKee and Harrison, 1995). Rose and Lee (1977) studied the interaction between *E. tenella* sporozoites and peritoneal cells from normal and *Eimeria*-immune chickens. The uptake of sporozoites by cells from *Eimeria*-immune birds was greater than that by naive birds. The nature of the cells involved in the sporozoite uptake, however, remains to be determined.

Cytokines and Chemokines

A number of chicken genes of immunological importance have been cloned (Table 1). These genes have properties similar to those of their mammalian counterparts, although their role in poultry immune system and in disease processes needs to be better studied.

Interferons were first described in chickens by Isaacs and Lindenmann (1957) and have been shown to have various immunomodulating effects on a wide variety of tissues. Interferons are classified into type I (α-,β-,ω-, and τ-IFN) and type II (IFN-γ). Type I IFN of chickens, designated IFN1 and IFN2, show a low sequence homology to mammalian IFN (Sekellick et al., 1994). The chicken gene encoding IFN-α has been cloned and its biological function studied by many laboratories (Digby and Lowenthal, 1995; Song et al., 1997). Effects of the pretreatment of chicken macrophages or fibroblasts with crude cultural supernatants containing IFN-γ on *E. tenella* sporozoites were examined in various in vitro systems (Lillehoj et al., 1989; Dimier et al., 1998; Lillehoj and Choi, 1998). The culture supernatants from ConA-stimulated spleen cells or from the virus-transformed cell line reticuloendotheliosis virus inhibited the intracellular development of *E. tenella* in the cells. Effects of the recombinant bovine and human IFN-γ on the invasion of sporozoites of *E. tenella* in cultures of bovine and human cell line (HEP-2), respectively, were examined (Kogut and Lange, 1989b).

Interferon-γ production during coccidiosis was examined using a quantitative RT-PCR (Choi et al., 1999; Yun et al., 2000c), and recently using gene expression profiling (Min et al., 2003). After *E. acervulina* infection, IFN-γ mRNA expression was detected in the cecal tonsils and spleen but not in the duodenum of SC chickens (Choi et al., 1999). In *E. tenella*-infected chickens, IFN-γ transcripts were detected in the spleens, cecal tonsils, and IEL follow-

ing the primary and the secondary infections with *E. tenella*. The marked increase in the transcripts of IFN-γ was shown at d 6 after primary infection in the cecal tonsils. Laurent et al. (2001) recently showed that IFN-γ expression in the cecum and jejunum of White Leghorn (PA12) chickens increased over 200-fold above the control at 7 d after primary infection with *E. tenella* and *E. maxima* using RT-PCR. The role of recombinant IFN-γ in coccidiosis was directly assessed by injecting the recombinant chicken IFN-γ produced in *Spodoptera frugiperda* insect cells transfected with a pcDNA vector carrying the chicken IFN-γ gene (Lillehoj and Choi, 1998). Multiple intramuscular injections (3 times) of the supernatant of *S. frugiperda* cells expressing recombinant chicken IFN-γ on 1 d prior to, and 2 and 4 d after infection with *E. acervulina*, conferred significant protection as measured by body weight loss and oocyst shedding in both SC and TK strains (Lillehoj and Choi, 1998). Furthermore, *E. tenella* sporozoites were inhibited from undergoing the intracellular development in a chicken cell line that is stably transfected with the chicken IFN-γ gene. The treatment of chicken cells with the recombinant chicken IFN-γ inhibited the intracellular development of *E. tenella* without affecting the sporozoite invasion of host cells (Lillehoj and Choi, 1998). These results provide the first direct evidence that chicken IFN-γ exerts an inhibitory effect against *Eimeria* and provides a rational basis for the use of this cytokine as a vaccine adjuvant against coccidiosis.

Interleukin-2 plays an important role in the function of the immune system. IL-2 is a potent growth factor for a variety of cell types including T cell differentiation, B cell development and NK cell activation (Lillehoj et al., 1992; Farner et al., 1997). Chicken IL-2 gene has been cloned (Sundick and Gill-Dixon, 1997) and its biological function characterized (Stepaniak et al., 1999; Choi and Lillehoj, 2000; Lillehoj et al., 2001). After the primary as well as the secondary infections with *E. acervulina*, a significant enhancement of IL-2 mRNA transcripts was observed in the spleen and intestine (Choi and Lillehoj, 2000). The protective effect of IL-2 on the recombinant vaccination of chickens with the 3-1E coccidia gene was recently demonstrated by DNA vaccination (Lillehoj et al., 2000; Min et al., 2001). Co-injection of the IL-2 gene with the 3-1E coccidia gene enhanced the host response to the recombinant vaccination.

Interleukin-16 was originally described as a lymphocyte chemoattractant factor synthesized by CD8+ and CD4+ T cells and released in response to antigens, mitogens, histamine, or serotonin (Cruikshank et al., 2000). Further analysis indicated that IL-16 is generated by B cells, mast cells, epithelial cells, macrophages, fibroblasts, and eosinophils (Laberge et al., 1999; Cruikshank et al., 2000; Kaser et al., 2000; Sharma et al., 2000). Initially, IL-16 is produced as a 67-kDa pro-IL-16 (Baier et al., 1997) that subsequently is cleaved by caspase-3, producing a 17 kDa-secreted form of the chemokine that aggregates to form biogically active homotetramers (Center et al., 1996; Zhang et al., 1998). IL-16 is chemoattractive for CD4+ T cells, eosinophils, and monocytes through a mechanism involving binding to CD4 (Zhang et al., 1998), although recent data suggest that CD4 is not the only receptor for IL-16 function (Mathy et al., 2000). In addition to its chemotactic function, IL-16 induces the expression of IL-2 receptor α and MHC class II molecules (Cruikshank et al., 1987; Center et al., 1996; Zhang et al., 1998; Mathy et al., 2000). In contrast, IL-16 inhibits TCR/CD3-dependent activation and promoter activity of the human immunodeficiency virus (HIV) (Cruikshank et al., 1996; Maciaszek et al., 1997). IL-16 expression has been implicated in inflammatory responses in atopic dermatitis (Laberge et al., 1998), asthma (Laberge et al., 1997), multiple sclerosis (Biddison et al., 1997), rheumatoid arthritis (Klimiuk et al., 1999), systemic lupus erythematosus (Lee et al., 1998), and bowel disease (Seegert et al., 2001).

The single copy gene encoding human and mouse IL-16 consists of 7 exons and 6 introns (Bannert et al., 1999), and homologous sequences have been cloned from nonhuman primates, cat, and cow (Bannert et al., 1998; Leutenegger et al., 1998; Mertens et al., 2000). Keane et al. (1998) showed cross-species structural and functional conservation between human and murine IL-16. In contrast, due to low sequence homology between avian and mammalian cytokines, most of the former, including IL-16, have yet to be isolated and characterized (Staeheli et al., 2001). Recently, using nucleotide sequence homology, a cDNA from an expressed sequence tag cDNA library, prepared from intestinal IEL of *Eimeria*-infected chickens and containing a full-length open reading frame (ORF) of pro-IL-16 encoding chicken pro-IL-16, was characterized (Min and Lillehoj, 2004). Interleukin-16 is a proinflammatory cytokine synthesized as a precursor protein (pro-IL-16). The encoded protein, predicted to consist of 607 amino acids, showed 86% sequence homology to duck pro-IL-16 and 49 to 52% homology to various mammalian homologues. By Northen blot analysis, IL-16 transcripts were identified in chicken lymphoid tissues but not in the nonlymphoid tissues examined. A recombinant protein containing the COOH-terminal 149 amino acids of pro-IL-16 when expressed in COS-7 cells showed chemoattractant activity for splenic lymphocytes.

Interleukin-17 was cloned originally from an activated T-cell hybridoma produced by the fusion of a mouse cytotoxic T-cell clone with a rat T-cell hybridoma and referred to as CTLA-8 (cytotoxic T lymphocyte-associated antigen 8) (Rouvier et al., 1993). Further analysis indicated that the IL-17 cloned by Rouvier et al. (1993) was derived from the rat T-cell hybridoma partner (Yao et al., 1995; Kennedy et al., 1996). Whereas the predicted amino acid sequence of IL-17 exhibited 57% identity to the HVS 13-gene product, the 3′ untranslated region of its mRNA contained nucleotide sequences associated with mRNA instability often found in transcripts of cytokines, growth factors, and proto-oncogenes (Shaw and Kamen, 1986; Schneider et al., 2000). IL-17 and its family subsequently were cloned from humans and mice and were shown to encode a cytokine associated with inflammation and immunity (Fossiez et al., 1996; Kennedy et al., 1996; Ag-

garwal and Gurney, 2002). IL-17 was produced in a mixture of glycosylated (22 kDa) and nonglycosylated (15 kDa) forms and secreted by activated CD4+ T cells as covalently bound homodimers (Fossiez et al., 1996). Whereas IL-17 transcripts were restricted to activated T cells, their receptors were found to be expressed ubiquitously in a variety of mammalian tissues and cell lines (Yao et al., 1995, 1997). Functional studies indicated that IL-17 is involved in a broad range of cellular activities. For example, IL-17 stimulated osteoclastogenesis (Kotake et al., 1999), granulopoiesis (Schwarzenberger, et al., 1998), and T-cell proliferation by suboptimal concentrations of phytohemagglutinin (Yao et al., 1995). When CD34+ hematopoietic progenitor cells were co-cultured with irradiated fibroblasts in the presence of IL-17, the cells proliferated and differentiated into neutrophils (Fossiez et al., 1996). Some of these effects were undoubtedly related to the ability of IL-17 to upregulate synthesis and secretion of numerous cytokines and chemokines including IL-6, IL-8, granulocyte-colony-stimulating factor, and prostaglandin E2 (Fossiez et al., 1996). In addition, IL-17 augmented the secretion of IL-1β, IL-10, IL-12, IL-1 receptor antagonist, tumor necorsis factor (TNF)-α, and stromelysin by human peripheral blood macrophages (Jovanovic et al., 1998) and stimulated synthesis of NO by human osteoarthritis cartilage cells (Attur et al., 1997). Many of these activities were induced at the transcriptional level by regulatory factors such as NF-κB, AP-1, and cAMP-responsive element-binding protein (Yao et al., 1995; Jovanovic et al., 1998). Through stimulation of cytokine/chemokine synthesis, IL-17 has been implicated in rheumatoid arthritis (Chabaud et al., 1999; Ziolkowska et al., 2000), obstructive airway disease (Linden et al., 2000), gastritis (Luzza et al., 2000), and allograft rejection (Van Kooten et al., 1998; Antonysamy et al., 1999).

Interleukin-17 is a proinflammatory cytokine produced by activated T cells. Min and Lillehoj (2002) cloned a 917 bp cDNA encoding the IL-17 gene from an expressed sequence tag cDNA library prepared from intestinal IEL of *Eimeria*-infected chickens. It contained a 507-bp ORF predicted to encode a protein of 169 amino acids with a molecular mass of 18.9 kDa, a 27-residue NH2-terminal signal peptide, a single potential N-linked glycosylation site, and 6 cysteine residues conserved with mammalian IL-17. Chicken IL-17 shared 37 to 46% amino acid sequence identity with the previously described mammalian homologues and also was homologous with the ORF 13 of Herpes virus saimiri (HVS 13). By Northen blot analysis, IL-17 transcripts were identified in a reticuloendotheliosis virus-transformed chicken lymphoblast cell line (CU205) and ConA-stimulated splenic lymphocytes but not other chicken cell lines or normal tissues. Conditioned medium from COS-7 cells transfected with chicken IL-17 cDNA induced IL-6 production by chicken embryonic fibroblasts suggesting a functional role for the cytokine in avian immunity.

Neither TNF-α nor -β has been well characterized in poultry at present. However, macrophages obtained during and immediately following an infection with *E. maxima* or *E. tenella* produced a TNF-like factor in a biphasic fashion whereby the first peak was associated with the pathogenesis of disease and the second peak with the development of a protective immunity (Byrnes et al., 1993). The production of significantly greater amounts of TNF during the 3 to 6 d after inoculation correlates with the appearance of the most characteristic local and systemic pathophysiological changes induced by coccidia (Byrnes et al., 1993). Zhang et al. (1995a,b) investigated the effect of a TNF-like activity on the pathogenesis of coccidiosis in inbred chickens. TNF-like factor was produced by the peripheral blood macrophages in a time- and dose-dependent manner following the primary, but not the secondary, *E. tenella* infection. Treatment of chickens with antibody against TNF resulted in a partial abrogation of *E. tenella*-induced body weight loss in SC chickens. Increased in vivo production of TNF-like factor in *E. tenella*-infected chickens in response to lipopolysaccharide was also reported (Smith and Ovington, 1996).

Transforming Growth Factor (TGF)-β is a pleiotropic anti-inflammatory cytokine that stimulates the repair of damaged mucosal epithelial integrity following injury (Robinson et al., 2000). Lymphocytes that secrete TGF-β downregulate the host immune and inflammatory responses, especially in the intestinal mucosa (Strober et al., 1997). The expression of TGF-β 2, 3, and 4 was investigated using cDNA probes and antibodies specific for the different TGF-β isoforms in chickens (Jakowlew et al., 1997). After infection with *E. acervulina*, the expression of TGF-β4 mRNA increased 5- to 8-fold in the intestinal IEL and 2.5-fold in the spleen, whereas the expression of mRNA for TGF-β2 and TGF-β3 remained constant in these cells. In a similar model of intracellular parasitic infection in mammals, in vitro stimulation of splenocytes from severe combined immune-deficient (SCID) mice with heat-killed *Toxoplasma gondii* resulted in the production of low levels of IFN-γ and 2- to 3-fold increase in the levels of TGF-β in culture supernatants (Hunter et al., 1995). Those mice were protected from *T. gondii* via IL-12 stimulation of IFN-γ production by NK cells. Blocking of TGF-β by the anti-TGF-β antibody resulted in a 3- to 4-fold augmentation in the IFN-γ production by the SCID mice splenocytes. Stimulation of the splenocytes from the SCID mice with IL-12 in combination with TNF-α or IL-1β resulted in the production of high levels of IFN-γ. Addition of TGF-β to these cultures inhibited production of IFN-γ in a dose-dependent manner. Administration of TGF-β to *T. gondii*-infected SCID mice resulted in an earlier mortality and shortening of the survival time of mice given the exogenous IL-12. Administration of anti-TGF-β to SCID mice beginning 4 h prior to the infection and every 2 d thereafter prolonged the survival time significantly. These data demonstrated the ability of TGF-β to antagonize the IL-12-induced IFN-γ production by SCID mice and suggested a role for TGF-β in the regulation of T-cell-independent resistance mechanism to *T. gondii* (Hunter et al., 1995).

In a mouse model of an experimentally induced acute toxoplasmosis, serum IFN-α/β increased gradually after

infection, but the IFN-γ was not detected in the systemic circulation at any time during infection (Diez et al., 1989). It was also observed that IFN-α/β and IFN-γ production were inversely correlated, suggesting that IFN-α/β production is an important factor associated with the acute toxoplasmosis-induced immunosuppression (Diez et al., 1989). In C57BL/6 mice infected with *Plasmodium yoelli*, the recombinant hybrid human IFN-α (which crossreacts with murine cells) reduced the blood parasite load and the hepatosplenomegaly induced by blood-stage parasites, although it did not inhibit the development of the parasite in the liver (Vigario et al., 2001). The reduced blood parasite load in the IFN-α-treated mice was associated with the reduction of erythropoiesis and reticulocytosis. The effect of IFN-α on *Eimeria* parasites was studied using recombinant bovine IFN-α (Kogut and Lange, 1989a). The intracellular development of *E. tenella* in the Madin-Darby bovine kidney cells, already treated with recombinant bovine IFN-α-1 for 24 h before infection and for 48 h after infection, was not affected although a similar treatment with recombinant bovine IFN-γ induced a significant reduction in the number of the total intracellular parasites as compared with the untreated controls (Kogut and Lange, 1989a). Later studies using recombinant chicken IFN-α also failed to demonstrate any inhibitory effect of *E. tenella* growing in various chicken cell lines such as HD11 (macrophages), DU24 and CHCC-OU2 (fibroblast cells), and LMH (hepatic epithelial cells) (Heriveau et al., 2000). Simultaneous injection of chickens with the cDNA encoding chicken IFN-α did not show any inhibitory effect on the parasites when the chickens were vaccinated with the 3-1E gene encoding a 21 kDa surface protein of *E. acervulina* (Min et al., 2001).

Interleukin-6 is a pleotropic lymphokine originally described as a T-cell-derived lymphokine; it induces the final maturation of B cells into antibody-producing cells (Narazaki and Kishimoto, 1994). Chicken IL-6 shows about 35% sequence identity with human IL-6 (Schneider et al., 2001). Bacterially expressed chicken IL-6 carrying a histidine tag in place of the signal peptide was biologically active and induced the proliferation of the IL-6-dependent murine hybridoma cell line 7TD1 (Schneider et al., 2001). Production of chicken IL-6-like factor activity was detected by a murine IL-6 7TD1 bioassay in serum taken from chickens infected with *E. tenella* during the course of a primary infection (Lynagh et al., 2000). IL-6 activity was detected during the first few hours postinfection indicating a possible role of IL-6 in the development of acquired immunity.

Interleukin-1 in vitro production by macrophages obtained from *Eimeria*-infected chickens was observed during and immediately following the infection with *E. maxima* or *E. tenella* (Byrnes et al., 1993). Lymphocytes from *Eimeria*-infected chickens produced a higher level of IL-1 following stimulation than did cells from noninfected birds. RT-PCR measurement of IL-1 production demonstrated a 27- to 80-fold increase in the IL-1β transcript level 7 d after infection with *E. tenella* and *E. maxima* (Laurent et al., 2001). The precise role of IL-1 in the development of resistance against coccidiosis needs to be better characterized in view of its documented role in various infections.

CC and CXC Chemokines

Chemokines are important mediators of cell migration during inflammation and in normal leukocyte trafficking. These proteins are generally active at nanomolar concentration and are produced by a wide variety of cell types in response to exogeneous irritants and endogeneous mediators such as IL-1, TNF, platelet-derived growth factor (PDGF), and IFN-γ (Oppenheim et al., 1991). Chemokines are structurally related heparin-binding proteins acting on various inflammatory cell types (Oppenheim et al., 1991). Chemokines are grouped into 4 structural families characterized by the position of their amino-terminal cysteine residues (Zlotnik and Yoshie, 2000). The CXC class, which has one amino acid separated by 2 cysteine residues, and the CC class, which has 2 consecutive cysteine residues, are the most common chemokines. IL-8 and K60 are CXC chemokines (Kaiser et al., 1999; Sick et al., 2000) and K203 is a CC chemokine recently cloned from chickens (Sick et al., 2000). The K203 cDNA, cloned from the chicken macrophage cell line HD-11 stimulated with lipopolysaccharide, revealed a 50% sequence identity to the mammalian macrophage inflammatory protein 1β (MIP-1β) (Sick et al., 2000). Laurent et al. (2001) showed that mRNA level of the CC chemokines, K203 and MIP-1β, were up-regulated 200- and 80-fold, respectively, in the cecum in response to *E. tenella* infection, and 100- and 5-fold in the jejunum in response to *E. maxima* infection. Interestingly, no discernible changes were observed in the mRNA levels of the CXC chemokine, IL-8, and K60.

Lymphokines and Cytokines for Immunomodulation

Crude cell-free culture supernatants from Marek's disease herpes virus-transformed lymphoblastoid cell lines (JMV-1), ConA-stimulated normal spleen cells, or sporozoite-stimulated immune T cells contain one or more lymphokine-like factors that enhanced protection against *Eimeria* (Lillehoj et al., 1989). In vitro treatment of avian macrophages showed an inhibitory activity against *Eimeria* growth in the cells (Lillehoj et al., 1989). Subsequent studies (Keller et al., 1992) on the immunologic effects of the cell-free culture supernatants of JMV-1 cells and their phenotypic characterization using a panel of mAb showed that this cell line is a T-helper cell line exhibiting a Class II MHC antigen. The JMV-1 cell line was not a killer or cytotoxic T cell line, even though it demonstrated in vitro NK cell cytotoxicity against the avian tumor cell line, LSCC-RP9, in a 4-h assay. Culturing spleen cells in the presence of JMV-1 supernatant for 4 h stimulated a significant increase in NK cell populations and the MHC class II Ia-bearing cell populations. These findings suggest that the protective effects of JMV-1 cell line and its cell-free supernatant against the parasites and Marek's disease

virus-induced diseases are due in part, to the lymphokine activation of effector cells (Keller et al., 1992).

Min et al. (2001) assessed the effects of directly injecting genes encoding the chicken cytokines IL-1β, IL-2, IL-8, IL-15, IFN-α, IFN-γ, TGF-β4, or lymphotactin on chickens vaccinated with the pcDNA carrying the 3-1E gene. Chickens were subcutaneously injected twice with 50 μg of the pcDNA3-1E vaccine plus a cytokine expression plasmid 2 wk apart and challenged with *E. acervulina* 1 wk later. IFN-α (1 μg or 10 μg of lymphotactin gene), when given simultaneously with the pcDNA3-1E vaccine, significantly protected chickens against body weight loss induced by *E. acervulina* infection. Parasite replication was also significantly reduced in chickens immunized with the pcDNA3-1E vaccine and co-injected with 10 μg of the IL-8, lymphotactin, IFN-γ, IL-15, TGF-β4, or IL-1β gene as compared with chickens given the pcDNA3-1E vaccine alone (Min et al., 2001). Flow cytometric analysis of the duodenal IEL showed a significant increase in CD3+ cells in chickens inoculated simultaneously with the pcDNA3-1E vaccine and the IL-8 or IL-15 expression plasmids in comparison with those of chickens inoculated with a suboptimal dose of pcDNA3-1E alone or in combination with the other cytokine genes tested (Min et al., 2001). These results indicate that the type and dose of cytokine genes injected into chickens influence the quality of the local immune response to DNA vaccination against coccidiosis.

In conclusion, host immune responses to protozoan parasites are extremely complex and involve many different effector mechanisms depending on the prior host exposure to the parasites, the stage of parasite development, the nutritional status of infected chickens, and the genetic makeup of the host. Development of a logical and efficient control strategy against parasitic diseases will depend on continued basic research to ascertain the detailed processes underlying protective immunity and pathogenesis induced by *Eimeria* parasites. Vaccination is a feasible means of controlling coccidiosis because *Eimeria* infections in natural hosts induce strong protective immunity that develops quickly. Although live virulent organisms, live attenuated strains, non-infective parasite derivatives, and genetically engineered subunit vaccines have been utilized to induce protection against avian coccidiosis, more research is needed in order to make any of these strategies practical for field conditions. The efficacy of future recombinant coccidia vaccines will depend on the identification of antigens displayed at different life cycle stages and the availability of vectors and delivery systems to elicit intestinal immune responses. Also, the existence of antigenic variations among *Eimeria* field strains warrants careful evaluation of the type of parasite antigens used in recombinant subunit vaccines. The advent of new genomics and molecular techniques to manipulate the genomes of host and pathogens alike and an enhanced knowledge of intestinal immune system will enable new approaches to vaccination against enteric pathogens to be realized within the near future. Furthermore, there is a need to understand the interactions of nutrition, infection, and immunity to explore new avenues for the development of cost-effective control strategies against coccidosis that do not rely upon chemical prophylaxis.

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